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Reconsideration of *S*-genotype for a Japanese Pear 'Kumoi'

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Summary

A Japanese pear 'Kumoi' was previously determined as S_3S_4 by pollination tests, but its *S*-genotype was reconsidered following our PCR-RFLP (S_1 to S_9) analyses and pollination tests. Based on its compatibility with 'Seigyoku' (S_3S_4), and PCR-RFLP analysis, 'Kumoi' was classified as S_1S_3 for the first time. Additional pollination tests were necessary to prove our contention, but 'Kumoi' did not supply sufficient flowers. 'Sekaiichi' was also assigned as S_1S_3 by PCR-RFLP analysis, and incompatibility with 'Kumoi'. Instead of 'Kumoi', 'Sekaiichi' was pollinated with the pollen from an S_3-homozygote and that from an S_4^{sm}-homozygote. The lack of fruit set revealed that 'Sekaiichi' was incompatible with the S_3 and S_4^{sm} pollen, leading us to predict that the *S*-genotype of 'Sekaiichi' was S_1S_3 or S_3S_4. Two *S*-genotypes with S_1S_3 and S_2S_3 segregated in hybrid progenies between 'Doitsu' (S_1S_2) and 'Sekaiichi', indicating that S_1 was present in 'Sekaiichi'. These results of pollination tests with 'Sekaiichi' indicated the *S*-genotype of 'Kumoi' was S_1S_3.

Key Words: Japanese pear, *Pyrus pyrifolia*, self-incompatibility, *S*-genotype, *S-RNase*.

Introduction

Japanese pear (*Pyrus pyrifolia* Nakai) exhibits gametophytic self-incompatibility that is controlled by a single *S*-locus with multi-alleles. *S*-genotype assignments have been used as an aid for both the breeding and management of pollination in orchards. Terami et al. (1946) identified seven *S*-alleles (S_1 to S_7) and classified 23 cultivars into 10 *S*-genotypes. Using these *S*-genotype assignments as first cross indicators, the *S*-genotypes have been determined for almost 40 cultivars (Hiratsuka et al., 1998; Machida et al., 1982; Ogaki, 1958). The present cross indicators do not cover the 21 *S*-genotypes having a combination of seven alleles. Pollens derived from *S*-homozygotes make *S*-genotype assignments easy. Three *S*-homozygotes, '312-9' (S_2S_2), '312-6' (S_3S_3), and 'Nashi chuukanbohon nou 1 gou' ($S_4^{sm}S_4^{sm}$) are selected from selfed progenies of 'Choujuurou' (S_2S_3) and self-compatible cultivar 'Osa-Nijisseiki' ($S_2S_4^{sm}$ (sm represents stylar-part mutant)). The three trees have been used as single allele indicator for S_2, S_3 and S_4 (Sato et al., 1991; Terai et al., 1999). Recently 'Doitsu' (S_1S_2) was found to be compatible with the pollen from 'Nijisseiki' (S_2S_4) but incompatible with the pollen from 'Osa-Nijisseiki' ($S_2S_4^{sm}$), revealing that the S_4^{sm} pollen recognizes not only S_4 but also S_1 (Saito et al., 2002).

The *S*-allele of Japanese pear encodes *S*-RNase as a pistil product (Ishimizu et al., 1996; Sassa et al., 1992). Based on the nucleotide sequences of S_1- to S_7-*RNase*, a PCR-RFLP (S_1 to S_7) system has been established for rapidly assigning the *S*-genotype in Japanese pear cultivars harboring S_1- to S_7-allele; Genomic PCR with *S*-allele-specific primers provided S_1- to S_7-amplicon (product), which are discriminated by following digestions with six *S*-allele-specific restriction endonucleases (Ishimizu et al., 1999). Recently, two new *S*-RNase genes, S_8- and S_9-*RNase*, have been cloned from some cultivars for which this system could not be adapted. Hence, a new PCR-RFLP (S_1 to S_9) system has been developed for *S*-genotype assignments in Japanese pear cultivars harboring S_1 to S_9 (Castillo et al., 2002; Takasaki et al., 2004).

Using PCR-RFLP system and pollination tests, we revised four *S*-genotypes, 'Akaho' (S_3S_5), 'Tanzawa' (S_4S_5), 'Ichiharawase' (S_1S_8), and 'Meigetu' (S_1S_8) among the first cross indicators (Castillo et al., 2001). These revisions raised some doubt about the *S*-geno-

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types determined by these indicators.

'Kumoi' (Nashi nourin 1 gou) is a hybrid of 'Ishiiwase' and 'Yakumo' (S_1S_4) (Kajiura, 1955); it was determined as S_3S_4 by incompatibility with 'Tanzawa' (S_3S_4) (Ogaki, 1958). However, the S-genotype of 'Tanzawa' has been revised to S_4S_5 as described above. In this paper, we assigned the S-genotype of 'Kumoi' using the PCR-RFLP (S_1 to S_9) system, and confirmed its S-genotype with pollination tests.

Materials and Methods

Plant materials

Eight Japanese pears; 'Kumoi', 'Tanzawa', 'Seigyoku', 'Ishiiwase', 'Yakumo', 'Sekaiichi', 'Okusankichi', and 'Doitsu', and two S-homozygotes; '312-6' (S_3S_3) and 'Nashi chuukanbohon nou 1 gou' ($S_4^{sm}S_4^{sm}$), were employed in this study. All trees were planted at the National Institute of Fruit Tree Science (NIFTS), National Agriculture and Bio-oriented Research Organization in Tsukuba, Ibaraki, Japan. 'Yakumo' and 'Doitsu' were pollinated with pollen from 'Sekaiichi', and hybrid seeds were obtained.

PCR-RFLP (S_1 to S_9) analysis

PCR-RFLP (S_1 to S_9) analysis was performed according to the procedure described by Takasaki et al. (2004). Genomic DNA was extracted from young leaves of each cultivar or embryos of the hybrid seeds by a cetyltrimethylammonium bromide method (Doyle and Doyle, 1987). PCR was conducted by using the Expand High-Fidelity PCR system (Roche Diagnostics) for the amplification of S-alleles. Genomic DNA (about 50 ng) was mixed with 0.3 μM 'FTQQYQ' primer (5′-TTTACGCAGCAATATCAG-3′), 0.3 μM 'anti-(I/T) IWPNV' primer (consists of a mix of 0.15 μM 'anti-IIWPNV' (5′-AC (A/G) TTCGGCCAAATAATT-3′), and 0.15 μM 'anti-TIWPNV' (5′-ACGTTTGGCCAAATAGTT-3′)), 200 μM dNTP, 1 × PCR-buffer, 1U Taq polymerase, and distilled water to make a final volume of 30 μl. PCR amplification was carried out for 10 cycles of denaturation for 15 sec at 94℃, annealing for 30 sec at 48℃ and extension for 2 min at 70℃, following 20 cycles of denaturation for 15 sec at 94℃, annealing for 30 sec at 48℃, and extension for 2.5 min at 70℃, with a final extension for 7 min at 70℃. PCR products were digested with the following S-allele-specific restriction endonucleases; *Sfc*I (S_1 specific) at 25℃, *Afl*II (S_2 specific), *Nru*I (S_8 specific), *Nde*I (S_4 specific), *Alw*NI (S_5 specific), *Hinc*II (S_6 specific), *Ppu*MI (S_3, S_5 specific), and *Acc*II (S_6, S_7 specific) at 37℃, and *Bst*BI (S_9 specific) at 65℃. PCR products and digested fragments were electrophoresed on 2% agarose gels.

Pollination tests

Pollination tests were performed at the orchards of NIFTS. About 15 clusters with 2 flowers at the balloon stage (1-2 days before anthesis) were carefully emasculated, pollinated with pollen of a cross indicator, then covered with paper bags to avoid contamination with a foreign pollen. Within 70 to 80 days after pollination, the number of fruit sets and viable seeds were counted. When fruit set was less than 30%, the cross was judged to be incompatible.

Results and Discussion

Terami et al. (1946) identified 'Tanzawa' as S_3S_5. Whereas, Ogaki (1958) assumed 'Tanzawa' to be S_3S_4 and suggested that 'Kumoi' was S_3S_4 being incompatible with pollen from 'Tanzawa'. A part of the confusion raised from these earlier studies was resolved by recent pollination tests and PCR-RFLP analyses. 'Tanzawa' is compatible with 'Housui' (S_3S_5) (Hiratsuka et al., 1998) but incompatible with 'Kousui' (S_4S_5) (Castillo et al., 2001), revealing that its genotype is not S_3S_5 but S_4S_5. Hence, its S-genotype was revised as S_4S_5. With this reversion, S-genotype of 'Kumoi' may also need to be revised to S_4S_5. However, 'Kumoi' was compatible with pollen from 'Tanzawa' (S_4S_5) and 'Seigyoku' (S_3S_4),

Table 1. Cross-compatibility of 'Kumoi' and 'Sekaiichi' with cross indicators in pollination tests.

Seed parent	Pollen parent	No. of pollinated flowers	No. of fruit set	Fruit set (%)	No. of Seeds per fruit	Compatibility[y]
Kumoi	Tanzawa (S_4S_5)[z]	34	26	76.5	8.7 ± 0.3	Compatible
Kumoi	Seigyoku (S_3S_4)	30	28	93.3	8.7 ± 0.2	Compatible
Kumoi	Sekaiichi	30	0	0	-	Incompatible
Sekaiichi	312-6 (S_3S_3)	30	0	0	-	Incompatible
Yakumo (S_1S_4)	312-6 (S_3S_3)	30	28	93.3	7.5 ± 0.5	Compatible
Sekaiichi	Nashi chuukanbohon nou 1 gou ($S_4^{sm}S_4^{sm}$)	30	0	0	-	Incompatible
Okusankichi (S_5S_7)	Nashi chuukanbohon nou 1 gou ($S_4^{sm}S_4^{sm}$)	30	28	93.3	4.1 ± 0.4	Compatible
Sekaiichi	Seigyoku (S_3S_4)	30	27	90.0	8.3 ± 0.3	Compatible

[z] Parenthesises show the S-genotype determined previously by pollination tests.
[y] Cross was considered incompatible when fruit set was less than 30% .

indicating that 'Kumoi' was neither S_4S_5 nor S_3S_4 (Table 1).

'Kumoi' and its parents, 'Ishiiwase' (not determined by pollination tests) and 'Yakumo' (S_1S_4), were analyzed by using PCR-RFLP (S_1 to S_9) system. PCR with 'FTQQYQ' and 'anti-(I/T) IWPNV' primers amplified products of ca. 1.3 kb and ca. 350 bp from these cultivars (Fig. 1). PCR product of ca. 1.3 kb from 'Ishiiwase' was digested with *Bst*BI, and ca. 350 bp with *Ppu*MI but not with *Alw*NI. Two products of ca. 350 bp from 'Yakumo' were digested with *Sfc*I and *Nde*I. Two products of ca. 350 bp from 'Kumoi' were digested with *Sfc*I and *Ppu*MI but not with *Alw*NI. These digestion patterns of PCR products assigned 'Ishiiwase', 'Yakumo', and 'Kumoi' as S_3S_9, S_1S_4, and S_1S_3, respectively (Table 2). The S_1S_3 of 'Kumoi' is one of the expected S-genotypes in the progenies between 'Ishiiwase' (S_3S_9) and 'Yakumo' (S_1S_4).

Using pollination tests, we tried to determine 'Kumoi' as S_1S_3. However, 'Kumoi' showed male sterility and could not supply sufficient flowers because the 'Kumoi' planting at NIFTS was a preservation line and was maintained as a small tree by training and pruning. PCR-RFLP (S_1 to S_9) analysis provided us a Japanese pear cultivar with S_1S_3, 'Sekaiichi', whose PCR products of ca. 350bp digested with *Sfc*I and *Ppu*MI but not with *Alw*NI (Fig.1, Table 2). 'Kumoi' was incompatible with pollen from 'Sekaiichi', indicating both cultivars have the same S-genotype (Table 1). By confirming that 'Sekaiichi' possesses S_1 and S_3, we can prove that the S-genotype of 'Kumoi' is S_1S_3.

Cross indicators with S_1S_3 or the pollen from S_1- and S_3-homozygotes were essential to determine 'Sekaiichi' to be S_1S_3. There was S_3-homozygote, '312-6' (S_3S_3), but no cross indicator with S_1S_3 and S_1-homozygote. 'Sekaiichi' was incompatible with the S_3 pollen from '312-6', but 'Yakumo' (S_1S_4) was compatible (Table 1), confirming that 'Sekaiichi' possessed S_3. Since 'Sekaiichi' was incompatible with the S_4^{sm} pollen from 'Nashi chuukanbohon nou 1 gou' but 'Okusankichi' (S_5S_7) was compatible (Table 1), it indicates that 'Sekaiichi' possesses either S_1 or S_4. These results proved that 'Sekaiichi' had S_3 and either S_1 or S_4. The compatibility between 'Sekaiichi' and 'Seigyoku' (S_3S_4) removed the possibility that 'Sekaiichi' was S_3S_4, and estimated that 'Sekaiichi' is S_1S_3 (Table 1).

To confirm the presence of S_1 in 'Sekaiichi', we analyzed 22 hybrid seeds of 'Yakumo' × 'Sekaiichi' and 23 seeds of 'Doitsu' × 'Sekaiichi' by using PCR-RFLP (S_1 to S_9) system. If 'Sekaiichi' possesses S_1, 'Yakumo' (S_1S_4) and 'Doitsu' (S_1S_2) should reject the S_1 pollen from 'Sekaiichi', but accept the S_3 pollen. The

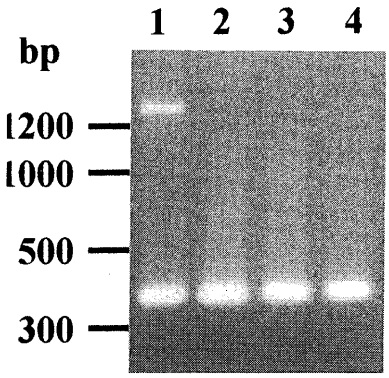

Fig. 1. Gel plate showing amplified *S-RNase* genes from genomic DNA of four Japanese pears by using PCR with primers 'FTQQYQ' and 'anti-(I/T) IWPNV'. 1: 'Ishiiwase', 2: 'Yakumo', 3: 'Kumoi', 4: 'Sekaiichi'.

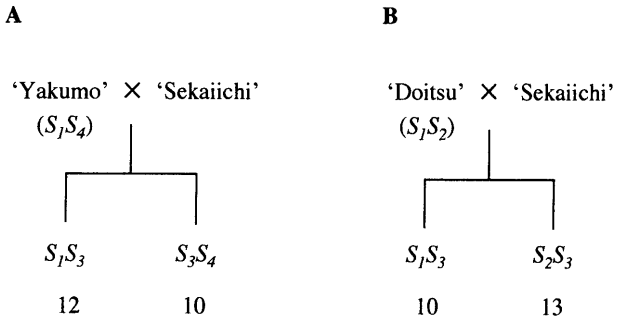

Fig. 2. S-genotype segregation patterns derived from hybrid seeds of 'Yakumo' × 'Sekaiichi' (A) and 'Doitsu' × 'Sekaiichi' (B). The number of hybrid seed is indicated under each S-genotype.

Table 2. S-genotype assignments of four Japanese pears by using PCR-RFLP (S_1 to S_9) analysis.

Cultivar	S-allele-specific restriction endonucleases									PCR-RFLP S-genotype
	ca. 1.3 kb		435 bp	ca. 350 bp						
	Afl II S_2	*Bst* BI S_9	*Nru* I S_8	*Sfc* I S_1	*Nde* I S_4	*Alw* NI S_5	*Hinc* II S_6	*Ppu* MI S_3, S_5	*Acc* II S_6, S_7	
Ishiiwase	−	+	−	−	−	−	−	+	−	S_3S_9
Yakumo	−	−	−	+	+	−	−	−	−	S_1S_4
Kumoi	−	−	−	+	−	−	−	+	−	S_1S_3
Sekaiichi	−	−	−	+	−	−	−	+	−	S_1S_3

+: One of two *S-RNase* products digested with the restriction endonuclease.
−: None of *S-RNase* products digested with the restriction endonuclease.

hybrid seeds of 'Yakumo' × 'Sekaiichi' possessed 12 S_1S_3 and 10 S_3S_4 seeds, whereas those of 'Doitsu' × 'Sekaiichi' exhibited 10 S_1S_3 and 13 S_2S_3 seeds (Fig. 2). These segregations with two S-genotypes indicate the presence of S_1 in 'Sekaiichi'.

In conclusion, our PCR-RFLP analyses, pollination tests and S-genotype segregations determined 'Sekaiichi' to be S_1S_3, leading us to revise S-genotype of 'Kumoi' to S_1S_3. With the S-genotype revision of cross indicators, S-genotypes determined by using these indicators should be re-examined by PCR-RFLP analysis and pollination.

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ニホンナシ‘雲井’のS遺伝子型の再検討

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摘　要

以前の交配試験でS_3S_4遺伝子型と決定されていたニホンナシ‘雲井’の遺伝子型を，PCR-RFLP (S_1~S_9) 分析および交配試験により再検討した．‘雲井’は‘清玉’(S_3S_4) と交雑和合性を示したので，S_3S_4ではないことが判明した．PCR-RFLP (S_1~S_9) 分析により，‘雲井’の遺伝子型はまだ報告されていないS_1S_3と推定された．‘雲井’がS_1およびS_3対立遺伝子を持つことを示すためには更なる交配が必要であったが，‘雲井’は十分な交配花数を供給できなかった．‘世界一’はPCR-RFLP (S_1~S_9) 分析によりS_1S_3と推定され，‘雲井’と交雑不和合性を示した．そこで，‘雲井’の代わりに‘世界一’にS_3ホモ個体の花粉と，S_4だけではなくS_1対立遺伝子も認識することができるS_4^{sm}ホモ個体の花粉を交配した．‘世界一’はS_3花粉とS_4^{sm}花粉の両方と交雑不和合性を示したことから，その遺伝子型はS_1S_3あるいはS_2S_4と予測された．PCR-RFLP (S_1~S_9) 分析から‘独逸’(S_1S_2)×‘世界一’の雑種種子はS_1S_3とS_2S_3の2つの遺伝子型に分離することが示され，‘世界一’におけるS_1対立遺伝子の存在が確認された．以上の結果から‘世界一’がS_1とS_3対立遺伝子を持つことが明らかになり，‘世界一’と交雑不和合性を示す‘雲井’のS遺伝子型はS_1S_3に修正すべきであると考えられた．